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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Boyd

Group Art Unit: 1648

Application No. 09/427,873

Examiner: J. Parkin

Filed: October 27, 1999

For: METHODS OF USING CYANOVIRINS TO INHIBIT VIRAL INFECTION

DECLARATION UNDER 37 C.F.R. § 1.132

1. I, Michael R. Boyd, am inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. The data set forth below were generated under my direction.

3. The nucleic acid sequence encoding wild-type cyanovirin is described in the present application (e.g., SEQ ID NO: 1). Replacement of Asn 30 with Ala, Gln or Val, and/or replacement of Pro 51 with Gly, as well as introduction of a penta-histidine epitope at the C-terminus of cyanovirin was carried out using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The resulting nucleic acids were inserted into gene transfer vectors and transfected into *E.coli*. Non-His-tagged recombinant proteins were purified from periplasmic fractions using reverse-phase liquid chromatography. His-tagged cyanovirins were purified by affinity chromatography with nickel-nitrilotriacetic acid resin under native conditions (Qiagene, Valencia, CA).

4. The stability of the CV-N homologs toward chemical denaturants was probed by tryptophan fluorescence and thermal stability by far-UV circular dichroism. The thermodynamic data are summarized in Table I, submitted herewith. Increased temperature stability was observed for the Pro51Gly and the Asn30Ala/Pro51Gly homologs.

5. To screen for antiviral activity, monocyte-tropic HIV-1 strains Ba-L and ADA and low-passage HIV-1 pediatric isolates RoJo and WeJo were obtained. All antiviral assays were performed on three-day-old phytohemagglutinin/IL-2 stimulated human peripheral blood mononuclear cells (PBMC) or six-day cultured monocytes. HIV-1 replication in PBMC (RoJo and WeJo) and monocyte/macrophage (Ba-L and ADA) cultures was determined by measurement of reverse transcriptase activity in the supernatant or by measurement of p24 antigen expression by ELISA, respectively. Antiviral data are reported as 50% effective concentrations (EC_{50}).

6. Results of the antiviral assays are presented in Table II, submitted herewith. Only non His-tagged cyanovirin homologs were screened. The spectrum of anti-HIV activity of the CV-N homologs against representative clinical isolates of HIV-1 was essentially the same as wild-type cyanovirin.

7. A triple-mutant cyanovirin homolog was generated by replacing Asn 30 with Gln, replacing Pro 51 with Gly, and replacing Ala 71 with Thr. In a primary anti-HIV screen employing HIV-1 IIIB virus in CEM-SS cells and using the methods described above, the triple mutant cyanovirin homolog had the same activity as wild-type cyanovirin.

8. In addition to the specific cyanovirin homologs described above, it is possible to generate peptides comprising 10 or more mutations in the cyanovirin amino acid sequence, e.g., peptides comprising 90% homology to the amino acid sequence set forth in SEQ ID NO: 2, which retain antiviral activity. Moreover, one of ordinary skill in the art has the ability to determine which amino acids of SEQ ID NO: 2 to alter to generate mutant cyanovirins having antiviral activity using routine laboratory techniques.

9. To identify amino acid residues appropriate for manipulation to generate a functional cyanovirin peptide, the ordinarily skilled artisan can determine the three-dimensional structure of the cyanovirin peptide from SEQ ID NO: 2. Indeed, my laboratory collaborated to elucidate the three-dimensional structure of cyanovirin, as reported in Bewley et al., *Nature Structural Biology*, *5*(7), 571-578 (1998) (submitted herewith). Ideally, mutations that do not modify the electronic or structural environment of the peptide are generated to retain optimal antiviral activity. By utilizing information regarding the three-dimensional structure of the peptide and the amino acid sequence of SEQ ID NO: 2, determination of which amino acids are critical for proper peptide folding by way of their location within the peptide structure or interaction with surrounding residues is within the skill of the ordinary researcher. For example, amino acid residues found in the β-strands of the cyanovirin peptide which are not involved in residue-residue binding interactions critical for three-dimensional structure are ideal targets for mutation to produce a cyanovirin homolog retaining proper overall topology.

10. In addition to creating mutations in regions of the SEQ ID NO: 2 that are not critical for three-dimensional structure, the ordinary researcher can determine which amino acid residues are likely responsible for viral binding. It is understood in the art that surface hydrophobicity plays a key role in protein-protein interactions, such as the interaction between cyanovirin and viral proteins. The ordinary researcher has the ability to map hydrophobic surface clusters on the cyanovirin peptide or cyanovirin homologs to predict regions critical for interaction with the viral envelope using routine methods such as those disclosed in Bewley et al. Amino acid residues not found in hydrophobic surface clusters are likely not critical for hydrophobicity of these clusters and, thus, are appropriate targets for mutation to create cyanovirin variants having 90% homology to SEQ ID NO: 2 and which retain antiviral activity.

11. With respect to screening for antiviral activity, the assays described in the detailed description and Examples of the present application are well within the skill of the ordinary researcher and require only routine laboratory techniques.

12. In addition, my laboratory has demonstrated the ability of cyanovirin (CV-N) to bind viral surface glycoproteins other than gp120 of HIV, namely the surface glycoprotein gp1-Z of the Ebola virus and the gC glycoprotein of the virus *Herpes simplex* (HSV). It has been further demonstrated that cyanovirins are able to bind to the carbohydrate moieties of viral surface glycoproteins from which the proteinaceous component has been removed (see Rule 132 Declaration submitted August 6, 2001). It is reasonable to predict based on the *in vitro* studies previously described that CV-N would inhibit infection by these viruses.

13. The surface glycoprotein (GP) of Ebola virus is central to the viral life cycle. Like HIV, the glycoprotein of Ebola virus is situated as spikes on the exterior of the virus which determine the specificity of cell-virus interactions resulting, in the case of Ebola virus, in preferential binding of the virus to endothelial cells. Also similar to HIV gp160, the Ebola virus glycoprotein undergoes post-translational proteolytic cleavage to form gp-1 and gp-2. Ebola virus gp-1, one of the glycoproteins described in the Rule 132 Declaration submitted August 6, 2001, is the putative surface receptor-binding glycoprotein of the virus, analogous to HIV gp120 (Ito et al., *Journal of Virology*, *75*, 1576-1580 (2001)). Likewise, glycoprotein C (gC) of the virus *Herpes Simplex* (HSV) is responsible for initial cell binding by HSV during the infection process (see, for example, Laquerre et al., *Journal of Virology*, *72*(12), 9683-9697 (1998)).

14. In that Ebola virus gp-1 and HSV gC, like HIV gp120, are responsible for binding of the virus to the cell surface, it is reasonable to predict that binding of CV-N to these glycoproteins inhibits the initial virus-cell contact required for viral infection. Accordingly, CV-N would be expected to inhibit Ebola virus and HSV infection.

15. Moreover, it has been demonstrated that CV-N effectively inhibits therapeutically or prophylactically Ebola virus infection in a mouse model developed for evaluation and prophylaxis and therapy of Ebola virus-induced hemorrhagic fever (Bray et al., *Journal of Infectious Disease*, *178*, 654-661 (1998)). The data set forth below were generated under my direction.

16. One group of mice, experimental Group 1, was injected intraperitoneally on day –1 through day 5 with 4.8 mg/kg CV-N. On day 0, mice of Group 1 were intraperitoneally inoculated with 100 plaque forming units (pfu) of Ebola virus. A second group of mice, experimental Group 2, was injected intraperitoneally with 100 pfu Ebola virus on day 0. Eight hours later, Group 2 mice were injected with 4.8 mg/kg CV-N, with administration of the antiviral protein continuing through day 5. Accordingly, Group 1 mice were administered CV-N prior to inoculation, and Group 2 mice were administered CV-N after inoculation. Both Group 1 and Group 2 mice were administered CV-N for five days following inoculation. A third group of mice, the control Group 3, did not receive CV-N, but also was inoculated with 100 pfu of Ebola on day 1. A dose of 100 pfu Ebola virus is known to produce 100% infectivity in this mouse model by intraperitoneal inoculation.

17. Due to the highly infectious nature of Ebola virus, the ability of CV-N to inhibit viral infection in animals at risk thereof, or to inhibit the course of viral infection in already infected-animals, was evaluated by prolonged survival of treated animals compared to untreated control animals. One hundred percent of the control group expired by seven days post-inoculation, with only 60% of control animals surviving at day 5.

18. In contrast, one hundred percent of Group 2 survived past day 7. In fact, twenty percent of Group 2 mice survived until day 9, with all animals expiring by day 11 post-infection. Even when initially administered after inoculation, the lethal

effects f Ebola virus infection were delayed by administration of CV-N, inferring that CV-N attentuated the spread of viral infection throughout the body.

19. Perhaps most significant, 100 percent of Group 1 animals survived until day 9, with 80% surviving until day 11, the last timepoint of the study. CV-N prevented the onset of symptoms of Ebola viral infection throughout the course of its administration and significantly delayed the lethal effects of the virus after administration of CV-N was stopped.

20. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 1/2/02

Michael R. Boyd, M.D., Ph.D.

Table I. Summary of Thermodynamic Parameters of CV-N Homologs

Protein (His-tag)	$T_{1/2}$ (°C)	$[GdnHCL]_{1/2}$ (M)	ΔG (kcal·mol^{-1})	m (kcal·mol^{-1}·M^{-1})
CV-N [a]	57.9 ± 0.5	1.6 ± 0.1	4.1 ± 0.2	2.6 ± 0.1
Pro51Gly	67.8 ± 0.5	4.4 ± 0.2	9.8 ± 0.5	2.2 ± 0.1
Asn30Gln/Pro51Gly [b]	68.3 ± 0.5	4.3 ± 0.2	9.4 ± 0.5	2.2 ± 0.1

a) For the non His-tagged CV-N: $T_{1/2} = 60.6 \pm 0.5$°C; $[GdnHCL]_{1/2} = 1.6 \pm 0.1$ M; $\Delta G = 4.1 \pm 0.2$ kcal·mol^{-1}; m = 2.6 ± 0.1 kcal·mol^{-1}·M^{-1}.

b) $T_{1/2}$ for Asn30Ala/Pro51Gly and Asn30Val/Pro51Gly are 67.5 ± 0.5°C and 68.7 ± 0.5°C, respectively.

Table II. Anti-HIV Activities of CV-N Homologs 50% REPRESENTATIVE CONC.

Compound	Virus Strain/Target Cells			
	RoJo/PBMC	WeJo/PBMC	Ba-L/Monocytes	ADA/Monocytes
	—	↑	↓	↓
CV-N	53.4±25.0	2.1±1.6 0.5-3.5	11.9±2.6	19.7±4.5
Asn30Ala	51.6±15.0	10.9±7.7 3-18	3.0±0.2	11.2±4.0
Asn30Gln	50.9±23.3	6.8±1.3 4-8	3.0±1.5	18.2±8.4
Asn30Val	48.2±12.9	5.4±2.8 2-8	4.1±1.2	6.1±2.6

CV-N 100 nM 50% INHIB
MUT-1 10 nM " 10× more effective